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Genetic and antigenic characterization of complete genomes of Type 1 Porcine Reproductive and Respiratory Syndrome viruses (PRRSV) isolated in Denmark over a period of 10 years[☆]



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ABSTRACT

Porcine Reproductive and Respiratory Syndrome (PRRS) caused by the PRRS virus (PRRSV) is considered one of the most devastating swine diseases worldwide. PRRS viruses are divided into two major genotypes, Type 1 and Type 2, with pronounced diversity between and within the genotypes. In Denmark more than 50% of the herds are infected with Type 1 and/or Type 2 PRRSV. The main objective of this study was to examine the genetic diversity and drift of Type 1 viruses in a population with limited introduction of new animals and semen. A total of 43 ORF5 and 42 ORF7 nucleotide sequences were obtained from viruses collected from 2003 to February 2013. Phylogenetic analysis of ORF5 nucleotide sequences showed that the Danish isolates formed two major clusters within the subtype 1. The nucleotide identity to the subtype 1 protogenotype Lelystad virus (LV) spanned 84.9–98.8% for ORF5 and 90.7–100% for ORF7. Among the Danish viruses the pairwise nucleotide identities in ORF5 and ORF7 were 81.2–100% and 88.9–100%, respectively. Sequencing of the complete genomes, including the 5'- and 3'-end nucleotides, of 8 Danish PRRSV Type 1 showed that the genome lengths differed from 14,876 to 15,098 nucleotides and the pairwise nucleotide identity among the Danish viruses was 86.5–97.3% and the identity to LV was 88.7–97.9%. The study strongly indicated that there have been at least two independent introductions of Type 1 PRRSV in Denmark and analysis of the full genomes revealed a significant drift in several regions of the virus.

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1. Introduction

Porcine Reproductive and Respiratory Syndrome (PRRS) is considered one of the most devastating and economically challenging diseases to the swine industry worldwide (Holtkamp et al., 2013). The causative agent is the PRRS virus (PRRSV), a small, enveloped single-stranded positive-sense RNA virus belonging to the *Arteriviridae* family, within the order *Nidovirales* (Cavanagh, 1997; Conzelmann et al., 1993; King et al., 2012). The PRRSV genome is approximately 15 kb in length with a 5' cap and a 3' polyadenylated tail and it encodes at least 10 open reading frames (ORFs) (Firth et al., 2011; Johnson et al., 2011; Snijder and

Meulenbergh, 1998; Wu et al., 2001). ORF1a and ORF1b covers the first three quarters of the PRRSV genome and encode two long non-structural polyproteins, pp1a and pp1ab, where the latter is expressed after a ribosomal frameshift (Snijder and Meulenbergh, 1998). pp1a and pp1ab are proteolytically cleaved into at least 12 non-structural proteins (nsps) (Snijder and Meulenbergh, 1998; Fang and Snijder, 2010). ORF2–5 encodes the membrane glycoproteins, GP2–GP5, and ORF6 and ORF7 encodes a non-glycosylated membrane protein (M) and the nucleocapsid (N) protein, respectively. Two small genes, ORF2b fully embedded in ORF2 and ORF5a partially overlapping ORF5 encodes the non-glycosylated proteins E and ORF5a protein (Wu et al., 2001; Johnson et al., 2011; Firth et al., 2011). The clinical signs of PRRS were first described in the late 1980s in pigs in North America and a few years later similar disease signs were reported in Europe (Keffaber, 1989; Wensvoort et al., 1991). Genetic nucleotide sequence comparisons of viruses isolated in Europe and North America respectively revealed only 50–60% identity between viruses; hence PRRSV was divided into two major genotypes, Type 1 and Type 2 (Allende et al., 1999). Genetic diversity is also pronounced within the genotypes, and

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Table 1
Overview of the 8 Danish Type 1 PRRSV complete genomes.

Virus	Accession no.	Collection year	Sequence material	Genome length ^a	Nt identity ^b (%)
DK-1992-PRRS-111.92	KC862566	1992	PAM 17. passage	15,098	92.0
DK-2003-6-5	KC862571	2003	PAM 1. passage	15,089	88.9
DK-2003-7-2	KC862572	2003	PAM 2. passage	15,074	88.7
DK-2008-10-5-2	KC862573	2008	Lung homogenate	14,876	94.8
DK-2010-10-10-3	KC862568	2010	PAM 1. passage	15,098	97.5
DK-2011-05-11-14	KC862567	2011	Serum	15,098	97.9
DK-2011-05-23-9	KC862569	2011	PAM 1. passage	15,098	97.2
DK-2012-01-05-2	KC862574	2012	Serum	14,876	96.0

^a Excluding the Poly(A) tail.

^b Pairwise nucleotide identity to Lelystad (M96262).

Type 1 has been divided into at least three subtypes based on the length of ORF7, where the protogenotype 1, the Lelystad virus (LV, Genbank: M96262), belongs to subtype 1 (Stadejek et al., 2002, 2006, 2008; Forsberg et al., 2002). While PRRSV Type 1, subtype 1 isolates are found in Central and Western Europe and globally, subtypes 2, 3 and the putative subtype 4 have only been found in Eastern European countries east of Poland (Stadejek et al., 2008).

In Denmark, PRRS was first diagnosed in March 1992 in a region with a high-density swine population in the southern part of Jutland close to the border of Northern Germany (Botner et al., 1994). Until 1996, only Type 1 PRRSV circulated in the Danish swine herds, but due to use of Type 2 Ingelvac PRRS MLV attenuated live vaccine that reverted back to high virulence in the field, Type 2 PRRSV was introduced into the Danish pig population (Botner et al., 1997; Madsen et al., 1998). Vaccination to control the disease caused by both PRRSV genotypes has since then been used in Denmark. Today, both Type 1 and Type 2 PRRSV are endemic with approximately 50% of the Danish herds being infected (www.spf-sus.dk). The vaccine Porcilis PRRS (MSD Animal Health) is the only licensed modified live vaccine against Type 1 PRRSV in Denmark. This vaccine was first introduced in Denmark in the beginning of 2001; however, use of this vaccine against Type 1 PRRSV was not consolidated until at the end of the decade. In Denmark it is only licensed for use in slaughter pigs, whereas in the rest of Europe it is also licensed for breeding animals. Extensive sequencing of circulating PRRSV isolates during the 1990s revealed that only subtype 1 of the Type 1 PRRSV strains was circulating in Denmark but only few Danish PRRSV isolates have been sequenced since then. The export of Danish pigs to Eastern and central Europe is significant i.e. in 2012 Denmark exported more than 9 million living pigs and more than ¼ of a million breeding animals whereas only few (less than 100) breeding animals were imported to Denmark. Despite this limited import of living pigs the possibility that foreign PRRSV isolates may be introduced into Denmark by contaminated transport carriers or persons cannot be excluded but this will probably be rare events. Nevertheless, the diversity of PRRSV circulating in Denmark is of mutual interest for a range of European pig producing countries. The main objective of this study was to close the gap in knowledge on the genetic diversity of currently circulating PRRS Type 1 viruses in Danish pigs and to investigate the genetic drift of the virus in a closed population with limited introduction of new strains. ORF5 and ORF7 sequences obtained from viruses isolated between 2003 and primo 2013 were examined. Furthermore, for the first time a complete genome analysis of Danish Type 1 viruses was performed and compared to other Type 1 complete genomes isolated in Europe, North America, and Asia. The results revealed that two viral clusters are circulating, one Lelystad-like and an almost unique 'Danish' cluster.

2. Materials and methods

2.1. Sample material

Lung tissues, serum, and nasal swabs included in this study were obtained from 27 Danish swine herds in the years 2003–2013. A number of viruses were proliferated in porcine alveolar macrophages (PAM) as previously described (Wensvoort et al., 1991). See Table 1 and Supplementary Table 1 for an overview of sample material.

Supplementary material related to this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.virusres.2013.10.009>.

2.2. RNA extraction

Total RNA was extracted from serum, nasal swabs, cell culture supernatant, or lung tissue. Lung tissue was prepared as a 5% homogenate in RLT buffer (QIAGEN) containing 1% β-mercaptoethanol (Sigma–Aldrich). RNA was extracted from lung homogenate and nasal swabs using RNeasy Minikit (QIAGEN) according to the manufacturer's instructions. Total RNA from serum and cell culture supernatant was purified using QIAamp Viral RNA Mini Kit (QIAGEN). Elution volume for both extractions methods was 60 µl. The RNA was stored at –80 °C until use.

2.3. Real time RT-PCR

Purified RNA was initially screened for PRRSV using a modification of the Primer Probe Energy Transfer RT-PCR (PriProET-RT-PCR) assay described by Balka et al. (2009).

2.4. cDNA synthesis and PCR amplification

Full-genome length cDNA synthesis was performed by SuperScript® III First-Strand Synthesis System (Invitrogen) following the recommendations by the manufacturer except that the cDNA synthesis step was extended to 90 min. A poly(dT) RT-primer was used (5'-CAG GAA ACA GCT ATG ACA CCT GAT CTC TAG AAA CGT T(T)₃₈-3') as cDNA primer (Nielsen et al., 2003). PCR amplification of ORF2 to ORF7 was carried out using AccuPrime™ Taq DNA Polymerase High Fidelity Kit (Invitrogen). The PCR mixture was prepared as recommended by the supplier except that the amount of AccuPrime™ Taq High Fidelity was increased to 0.5 µl. The PCR cycling was as follows: [94 °C for 15 s], 45 cycles: [94 °C 15 s, 55 °C for 30 s, 68 °C for 60 s] then finalized with 68 °C for 5 min and cool down to 4 °C. PCR cycling was performed on a T3 Thermocycler (Biometra). PCR primers for amplifying ORF2 to ORF7 were from Diaz et al. (2006). The PCR products were analyzed by agarose gel

electrophoresis using E-gel 2% agarose gels (Invitrogen) and purified with High Pure PCR Product Purification kit (Roche).

Long range PCR amplification for full genome sequencing was performed using full-genome length cDNA as template with AccuPrime™ Taq High Fidelity kit as described by Kvisgaard et al. (2013).

2.5. Cycle sequencing and next generation sequencing

PCR products of ORF2 to ORF7 were sequenced by cycling sequencing using the Sanger method (Sanger et al., 1977). In total, 33 ORF5 sequences (accession nos. KC862504–33, KC862561, KF662722–23) and 32 ORF7 sequences (KC862534–60, KC862561, KF662724–27) were obtained. Three (3) sequences each covering ORF2 to ORF7 (KC862562–64) were assembled from 7 overlapping PCR products. Eight (8) full genome sequences (KC862566–69; KC862571–74, Table 1) were produced from long range PCR amplicons covering the full genome of PRRSV in two or four fragments and sequenced by next generation sequencing technologies. For a detailed description of the procedure and next generation sequencing see Kvisgaard et al. (2013). In short, equimolar concentrations of the PCR amplicons covering the full genome of PRRSV were prepared for sequencing on Roche/454 Genome Sequencer FLX+Titanium (LGC Genomics GmbH, Berlin, Germany), Illumina HiSeq2000 (ARK genomics The Roslin Institute, University of Edinburgh), or Ion Torrent GPM sequencer (DTU Multi-Assay Core (DMAC), Kgs. Lyngby, Denmark).

2.6. Data analyses

The contigs of ORF2 to ORF7 were produced from assembling the raw data obtained by cycle sequencing against the PRRSV Type 1 reference sequence Lelystad (LV) (Genbank: M96262) using the commercial software CLC Main Workbench v. 6.6.2 (CLC BIO, Aarhus, Denmark). Mapping of the reads obtained from the full genome sequencing was performed by the Burrows-Wheeler aligner (BWA) using the Aln algorithm for the Illumina HiSeq2000 data and the bwsw algorithm for Roche 454 FLX and Ion Torrent PGM sequencer data (Kvisgaard et al., 2013). Amino acid sequences were predicted from the nucleotide sequences using CLC Main Workbench v. 6.6.2. Nucleotide and amino acid sequences were aligned using MUSCLE (Multiple Sequence Comparison by Log-Expectation). Phylogenetic trees were constructed from Neighbor Joining Algorithm with Bootstrap: 1000 replicates (CLC Main Workbench v. 6.6.2, CLC BIO, Aarhus, Denmark).

The rate of synonymous (dS) and non-synonymous (dNS) substitutions in each ORF of the 8 full genome sequences were calculated by Synonymous Non-synonymous Analysis Program (SNAP) (<http://hcv.lanl.gov/content/sequence/SNAP/SNAP.html>) (Körber, 2000). Estimates of amino acid substitutions were determined by the method of dS-dNS rates: dS-dNS > 0, dS-dNS < 0, and dS-dNS = 0 representing tendencies for synonymous and non-synonymous variations, and neutral mutations, respectively.

Potential N-glycosylation sites were determined for GP2, GP3, GP4, and GP5 using NetNGlyc 1.0 Server (<http://www.cbs.dtu.dk/services/NetNGlyc/>). A potential glycosylation site was accepted when the potential score was above the minimum threshold of 0.5 and the agreement between nine neural networks ('the jury') was 9/9 with minimum ++ (= potential < 0.5 (threshold), += potential > 0.5, ++ = potential > 0.5 and Jury agreement (9/9), +++ = potential > 0.75 and Jury agreement) (<http://www.cbs.dtu.dk/services/NetNGlyc/output.php>).

3. Results

3.1. Genetic diversity of ORF5 and ORF7

A total of 43 ORF5 and 42 ORF7 nucleotide sequence data were obtained from 43 viruses collected from 2003 to February 2013. The nucleotide lengths of the ORF5 and ORF7 sequences were 606 and 387 nucleotides, respectively. A single virus (DK-2012-10-01-27/KC862521) had an ORF5 nucleotide length of 603. The nucleotide identity to the subtype 1 genotype Lelystad virus (LV) (M96262) spanned for ORF5 84.9–98.8% and for ORF7 90.7–100% and among the Danish viruses the pairwise nucleotide identities of ORF5 and ORF7 were 81.2–100% and 88.9–100%, respectively.

3.2. Phylogenetic analysis of ORF5 and ORF7

The nucleotide sequences from this study were aligned with previously sequenced Danish viruses (Forsberg et al., 2002) and representative Type 1 subtype 1, 2, and 3 viruses from Europe, Asia, and North America obtained from Genbank. Phylogenetic analysis of complete ORF5 nucleotide sequences showed that the Danish viruses formed two major clusters, designated Clade A and Clade G according to the nomenclature by Shi et al. (2010) (Fig. 1). The majority of the Danish viruses isolated in the period 2008–2013 were classified in Clade A representing Pan European, North American and Asian isolated viruses. The Danish viruses in this clade shared 95.2–98.8% and 95.7–99.8% pairwise nucleotide identity to LV and the Porcine vaccine virus, respectively. All previously sequenced Danish viruses isolated before 1999 (Forsberg et al., 2002) formed Clade G together with 12 Danish viruses isolated in the years 2003–2012. One virus with a 3 nucleotide deletion in ORF5 belonged to this clade. Interestingly, this clade contained only Danish viruses and three viruses from China isolated in 2009. The phylogenetic analysis of the Danish complete ORF7 sequences (Fig. 2) showed clustering similar to the ORF5. However, it is not possible to make a direct comparison between the two trees since not all viruses represented in the ORF5 tree were available in Genbank for the ORF7 analysis. One interesting observation was that virus DK-2011-05-23-2 was located in Clade G according to its ORF5 sequence but in Clade A according to its ORF7 sequence. This virus only shared 86.6% identity to LV in ORF5 but 99.5% in ORF7.

3.3. Amino acid analysis of GP5 and N proteins

Examination of the deduced amino acid sequences from ORF5 showed that 105 of the 201 positions in GP5 were conserved in all of the 60 Danish isolates (52.2% conserved sites). Fifty-two positions varied in more than one isolate, and 22 positions varied in more than 10 isolates. At position aa122 and aa172, the amino acid residues leucine/phenylalanine and glycine/aspartic acid, respectively were equally distributed, hence a consensus amino acid at these two positions was not possible to determine. Three potential N-glycosylation sites, N37, N46, and N53, were conserved in 90%, 100%, and 100% of the viruses, respectively. The glycosylation site N37 was not present in 6 Danish viruses isolated between 1992 and 2007 which is in accordance with this glycosylation site not being present in the Type 1 reference strain LV. The cysteine residue thought to be involved in heterodimer formation with the M protein at position 50 (Verheije et al., 2002) was conserved in all the Danish sequences. The previously predicted (Plagemann, 2004; Oleksiewicz et al., 2002) major neutralizing epitope (aa38–54) and epitope site ES13 (aa178–200) were conserved in the consensus sequence of Danish GP5 sequences. One of the highly variable positions (aa122) was located in a putative T-cell epitope (Diaz et al., 2009). The major neutralizing epitope located at the amino acid position aa38–54 was highly conserved in all the Danish viruses,

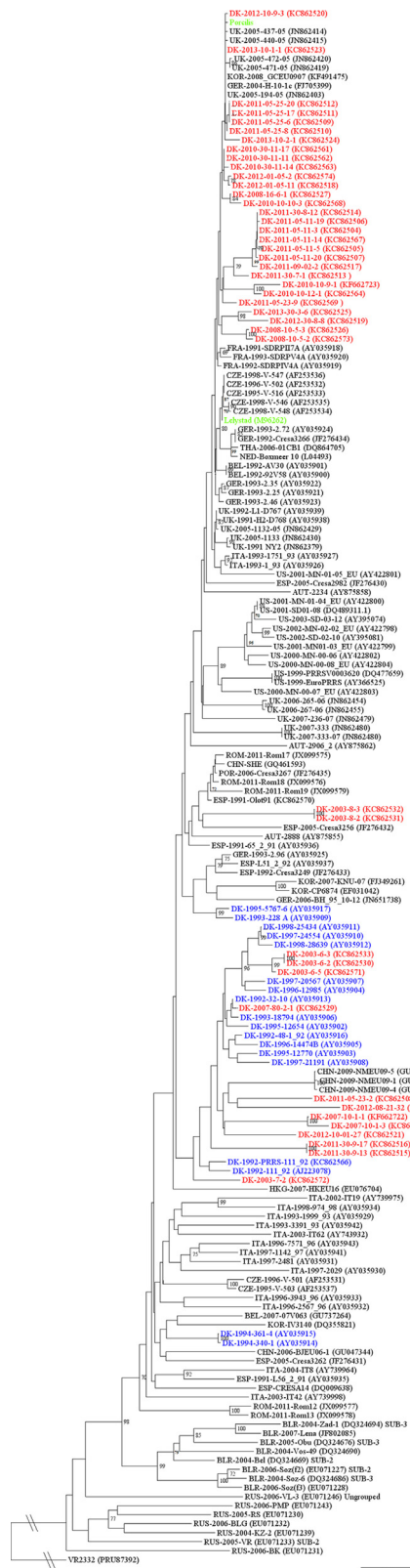


Fig. 1. Phylogenetic analysis of PRRSV Type 1 complete ORF5 nucleotide sequences. The Danish viruses isolated from 2003 to 2012 are shown in red. The Danish viruses isolated from 1992 to 1998 are shown in blue. The Porcine PRRSV vaccine and LV is shown in green. Bootstrap values in % are shown on branches. The scale bar represents 4 nucleotide changes per hundred.

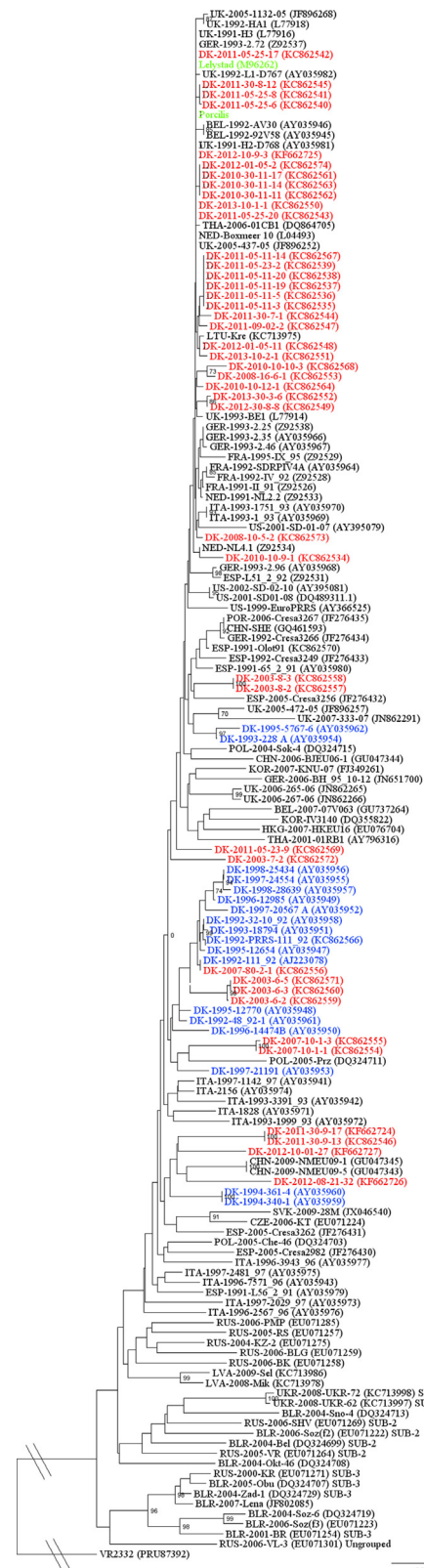


Fig. 2. Phylogenetic analysis of PRRSV Type 1 complete ORF7 nucleotide sequences. The Danish viruses isolated from 2003 to 2012 are shown in red. The Danish viruses isolated from 1992 to 1998 are shown in blue. The Porcine PRRSV vaccine and LV is shown in green. Bootstrap values in % are shown on branches. The scale bar represents 4 nucleotide changes per hundred.

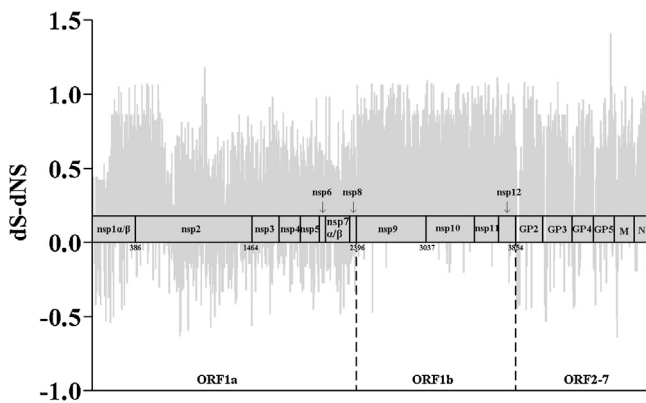


Fig. 3. Topology of synonymous (dS) and non-synonymous (dNS) substitutions in 8 Danish Type 1 viruses. Estimates of amino acid substitutions were represented by the method of dS-dNS rates: dS-dNS > 0, dS-dNS < 0, and dS-dNS = 0 representing tendencies for synonymous and non-synonymous variations, and neutral mutations, respectively. The numbering on the x-axis represents selected amino acid position in polyprotein 1a and polyprotein 1ab. aa386, aa1464, and aa3037 represent the putative cleaving sites between nsp1α/β and nsp2, nsp2 and nsp3, and nsp9 and nsp10, respectively in regards to the pp1ab sequence of LV.

which was also the case for the ES13 epitope (Oleksiewicz et al., 2002).

The examination of the deduced nucleocapsid protein revealed less variation between the Danish viruses compared to the GP5 protein. Eighty-eight out of 128 amino acid positions in the N protein were conserved (68.8% conserved sites). Twenty-five positions varied in more than one isolates, and only 5 positions varied in 10 or more isolates. The two cysteine residues at position 27 and 76 thought to be crucial for homodimer formation of the N protein were conserved throughout all the Danish isolates as was the putative linear B-cell epitope at position aa80–88 described previously (An et al., 2005). The four predicted T-cell epitopes (aa50–58, aa64–72, aa105–113, aa113–121) (Diaz et al., 2009) where highly conserved, with a minor variation at position aa64 where 10 isolates harbored an amino acid substitution. The most variable position in the N protein among the Danish viruses was at aa13, with 28 viruses harboring substitution.

3.4. Genetic analysis of complete genomes

Complete genomes, including the very 5' and 3'-end nucleotides, of 8 Danish Type 1 PRRS viruses isolated in the years 1992–2012 were analyzed and compared to published reference sequences. The genome lengths differed from 14,876 to 15,098 nucleotides (excluding the Poly(A) tail) and the pairwise nucleotide identity among the Danish viruses was 86.5–97.3% and the viruses were 88.7–97.9% identical to the LV strain (Table 1). The analysis of synonymous – non-synonymous (dS-dNS) mutations among the 8 Danish genomes showed a substantial higher amino acid substitution rate in ORF1a than in ORF1b (Fig. 3). The dNS mutations in the genes encoding the structural proteins were scattered throughout the genomes, with the highest rates in ORF3 and ORF5 (Fig. 3).

A global phylogenetic analysis of PRRSV Type 1 ORFs 1–7 (Fig. 4) showed that the Danish viruses divided into two clades as seen for the ORF5 and ORF7 phylogenetic analyses (Figs. 1 and 2). Five of the Danish viruses clustered with LV, Porcilis PRRS, and two viruses isolated in Germany (1992) and Thailand (2006). The three remaining Danish viruses grouped separately with a Chinese isolate from 2009.

3.5. The non-structural proteins

The pairwise nucleotide and amino acid identity of the non-structural proteins (nsp) of the Danish viruses were examined (for a complete overview of the pairwise identities of the different ORFs see Supplementary Table 2). The lowest average amino acid variation was found in nsp6, nsp11, nsp8, and nsp12, respectively. The highest variation was found in nsp1α/β and nsp2 with the amino acid identity range of 86.2–97.9% and 77.0–97.7% respectively. The predicted epitope site ES1 in nsp1α/β at position aa236–252 showed high variation with a substitution rate of 41% among the Danish viruses. A comparison of the predicted amino acid sequences of the nsp2 of the Danish isolates with 19 previous published sequences of Type 1 PRRSV viruses is shown in Fig. 5. The lengths of nsp2 for the Danish viruses varied from 1004 to 1078 amino acids. Interestingly, DK-2008-10-5-2 and DK-2012-01-05-2 had a 74 amino acid deletion also seen in the Porcilis PRRS strain (Darwich et al., 2011). These deletions spanned the predicted ES3 (Oleksiewicz et al., 2001) and T-cell epitope (Fang et al., 2004). Furthermore, a three amino acid deletion was discovered in DK-2003-6-5 located in ES7. The B cell epitope sites ES3, ES4, and ES7 together with the T-cell epitope were all located in highly variable regions, with substitution rates of 66%, 74%, 74% and 87%, respectively.

Supplementary material related to this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.virusres.2013.10.009>.

3.6. The structural proteins

Comparisons of the deduced amino acid sequences of the structural proteins of the Danish isolates revealed that the highest variation among the 8 Danish viruses was in GP3 and GP4 with an average similarity of 88.3 ± 4.3 and 90.4 ± 3.6 , respectively. Both GP3 and GP4 induce neutralizing antibodies (Oleksiewicz et al., 2001, 2002) and encode the B cell epitopes ES11 and ES12 at position aa60–87 and aa59–70 relative to GP3 and GP4 of LV, respectively. The amino acid sequences of GP3 of the eight isolates were aligned with the GP3 of additional 27 Danish viruses isolated in 2010 and in the 1990s (Forsberg et al., 2001). The ES11 showed a substitution rate of 46% among the 35 Danish viruses. In comparison to the LV strain, DK-2003-7-2 had a deletion in GP3 of 8 amino acids at the position aa240–247. This deletion has previously been detected in three Asian viruses (GU047344, GU047345, and EU076704) and eight Danish viruses isolated in the 1990s also had 1–8 amino acids deletions in this area. This region has previously been found to contain a B-cell epitope (243-RKASLSTS-250) (Oleksiewicz et al., 2000) but this antigenic motif was not present in any of the viruses examined in the present study. The ORF3 and the ORF4 coding regions overlap and hence any deletion in this shared coding region is likely to affect both protein products, which also was the case for DK-2003-7-2 which with an 8 amino acid deletion in GP4 at the position aa58–65 in LV. The ES12 of GP4 is located in a region prone for deletions and besides that, it is also a highly variable region with a 100% substitution rate among the Danish viruses. Alignment of GP3 and GP4 can be found as Supplementary Figs. 1 and 2, respectively.

Supplementary material related to this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.virusres.2013.10.009>.

Examination of potential N-glycosylation sites revealed 2 putative N-glycosylation sites in GP2, 7 putative N-glycosylation sites in GP3 and 4 putative N-glycosylation sites in GP4. The results of nine neural networks predicted that both putative N-glycosylation sites in GP2 (N173 and N179) were glycosylated, although one virus (DK-2010-10-12-1) did not score a unanimous vote from the jury

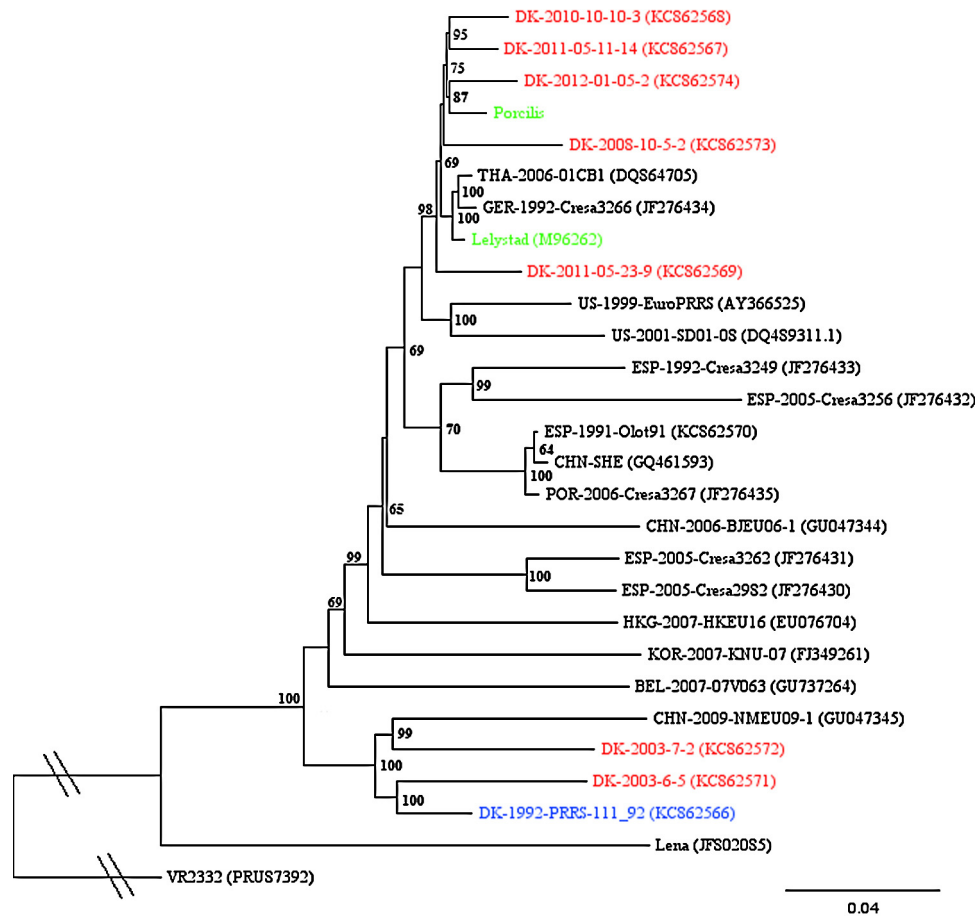


Fig. 4. Phylogenetic analysis of PRRSV Type 1 ORF1–7 nucleotide sequences. The Danish viruses isolated from 2003 to 2012 are shown in red. One Danish virus isolated in 1992 is shown in blue. The Porcilis PRRS vaccine and LV is shown in green. Bootstrap values in % are shown on branches. The scale bar represents 4 nucleotide changes per hundred.

network. For GP3 only N27, N50, N130, and N194 were potentially N-glycosylated. For N27, three Danish viruses were not predicted to harbor N-glycosylation at this site. For GP4 only N88 and N124 were predicted to be N-glycosylated.

Cysteine residues important for protein folding and function were 100% conserved in all the Danish viruses examined.

4. Discussion

There has been a gap in knowledge regarding the diversity of PRRSV Type 1 circulating in Denmark since the latest available information was obtained on viruses isolated in the late 1990s.

Most work on the diversity of European PRRS Type 1 viruses has focused on the structural proteins encoding genes, mainly ORF5, and ORF7 (Oleksiewicz et al., 2000; Forsberg et al., 2002). Here we report an extensive analysis of ORF5 and ORF7 of 43 viruses and full genome sequences of 8 Type 1 PRRS viruses isolated over almost two decades which to our knowledge makes this study the most extensive full-length Type 1 study published globally. The phylogenetic analyses of ORF5 and ORF7 revealed that the viruses isolated in Danish herds between 2003 and primo 2013 were highly diverse and clustered into two distinct phylogenetic clades, designated Clade A and Clade G in accordance to the definition proposed by Shi et al. (2010). Most of the viruses grouped with the Type 1 reference strain LV which is highly similar to the Porcilis vaccine strain, however a few recent viruses clustered into a phylogenetic clade that contained only the Danish viruses and these isolates were significant genetically different from the LV

strain. Notably, only viruses isolated after the Porcilis vaccine were introduced in Denmark grouped in the LV cluster which may indicate that the Porcilis strain may be circulating in the Danish pig herds. That the Danish PRRSV Type 1 viruses divide into two phylogenetic groups was also shown in the study by Forsberg et al. (2002). That study also indicated the existence of two Danish clusters in that two viruses isolated in 1993 and 1995 grouped with LV and were therefore called 'LV-like', although they only shared 93.6% pairwise identity to LV. LV was the first European PRRSV Type 1 isolated, and the sequence of this virus has been used as reference sequence in genetic comparisons, however based on the grouping in the phylogenetic tree not all the Type 1 viruses in Denmark may be progenies of LV (Shi et al., 2010). Accordingly, one of the first sequenced Danish PRRS viruses, DK111-92 (AJ223078) isolated in 1992, showed a pairwise nucleotide identity to LV of only 93.1% in ORF5. In the present study, the analysis of this Danish reference isolate (DK-1992-PRRS-111_92) is based on the 17th passage in cell culture, thus some of the observed difference may be caused by mutations induced from the propagation in cell culture. This may be regarded to have minor impact on the conclusions drawn, since the isolate is situated centrally in a clade of contemporary isolated viruses which have been sequenced directly from primary material or after only 1–2 passages in cell cultures. These analyses of early Danish PRRSV viruses suggested that more than one PRRSV Type 1 strain circulated in Europe in the early 90s and that Type 1 PRRSV was introduced into Denmark upon at least two different occasions. This is in accordance with results of a previous study that, based on analysis of ORF3, revealed that several viral lineages



Fig. 5. Alignment of the putative amino acid sequence of nsp2 for PRRSV Type 1. Epitope sites (ES) described by Oleksiewicz et al. (2001) are highlighted in black boxes. Gray squares represents deletions.

existed around the onset of the PRRSV epidemic in 1990 and dated the most common recent ancestor of these diverse strains to 10 years prior to the onset of the epidemic (Forsberg et al., 2001). The presence of diverse Type 1 isolates in Europe at such an early time point indicates, that Type 1 viruses have undergone a pronounced genetic drift in another species and were introduced into pigs at several occasions or that the virus has drifted in pigs a long time before clinical signs became evident. These findings are also in agreement with the observation that Type 1 PRRSV isolated in Italy and United Kingdom clustered in two groups, one LV like and one Italian-/British-like (Forsberg et al., 2002; Frossard et al., 2013; Shi et al., 2010).

Interestingly, one Danish virus, DK-2011-05-23-2, grouped in Clade G on the basis of its ORF5 sequence but in Clade A on the basis of its ORF7 sequence. The ORF5 and ORF7 sequences from this

virus were obtained from primary material and hence may indicate a double infection of the pig. One LV/porcilis like and one 'Danish-like'. Another explanation could be that this virus is a recombinant of the vaccine-strain and a 'Danish-like' virus. Unfortunately the virus failed to propagate in cell culture and therefore this could not be investigated further. Despite the high level of diversity among the Danish ORF5 nucleotide sequences, the deduced amino acid sequence of GP5 showed high level of conservation at the positions of putative N-glycosylation sites, cysteine residues important for heterodimer formation with M protein, and also the putative neutralizing epitope was well conserved throughout all the Danish viruses.

In conclusion, the analysis of the ORF5 and ORF7 sequences indicated the presence of circulating viruses belonging to two clades, one dominating vaccine/LV like and one resembling an early

introduced strain. Importantly, the analysis also confirmed that all the Danish isolates fall within genotype 1 subtype 1 and are distinct from the recently described Eastern European subtypes (Stadejek et al., 2008).

The sequencing of the complete genomes of Danish PRRS field viruses confirmed that there is a high overall diversity among Type 1 viruses in Europe. Thus, the pairwise nucleotide variation over the complete genome of the Danish viruses was 2.7–13.5%. Even among the most recently isolated viruses (2010–2012) the diversity was relatively high (2.7–5.8%). The variation within the viral genome showed to be unequally distributed as NSP1 α/β , NSP2, ORF3, and ORF4 showed the highest level of variation with 9.2%, 12.4%, 9.2%, and 9.7% average variation, respectively. The genomic regions with the lowest variation were ORF6 and ORF7 with 5.1% and 4.6% average variation, respectively. ORF1b showed a relatively high average nucleotide variation of 7.7%, however, most of the variations were synonymous, and consequently the average amino acid variation was only 3.1%. This level of amino acid conservation correlates well with the fact that this part of the viral genome encodes the key enzymes; RNA dependent RNA polymerase (nsp9) and helicase (nsp10) important for the RNA replication and unwinding of RNA duplexes, respectively (Fang and Snijder, 2010). The overall diversity of the Danish viruses fits very well with the global picture of Type 1 PRRSV diversity recently published by Darwich et al. (2011).

The nsp2 protein is the largest of the mature PRRSV proteins and has been shown to be critical in proteolytic processing of proteins active in virus replication and modulation of the immune response (Fang and Snijder, 2010; Han et al., 2009). The N-terminal of nsp2 possesses a putative cysteine protease domain of about 100 amino acids (aa36–aa140, Fig. 5). This cysteine domain is likely to be required for nsp2/3 proteolysis (Han et al., 2007). Nsp2 is the viral protein containing most B-cell epitopes (Oleksiewicz et al., 2001) and the antibody response to nsp2 is greater than toward any other PRRSV protein, however the antibodies raised against this protein is non-neutralizing (Johnson et al., 2007; Brown et al., 2009). One epitope, ES2, located in the putative cysteine protease domain, showed high level of conservation among the Danish viruses and globally, which agrees with the earlier findings that this domain is important for viral survival, as deletion of the domain is lethal to the virus (Han et al., 2007). The length of the nsp2 protein has been reported to be highly variable (Fang et al., 2004; Han et al., 2006) which also was the case for the Danish viruses in this study (1004–1078aa). DK-2008-10-5-2 and DK-2012-01-05-2 contained a 74 amino acid deletion at the exact same position as the Porcilis PRRS vaccine. However the pairwise nucleotide identity for ORF1-7 of these two Danish viruses and the vaccine strain was only 95.8% and 97.4%, respectively showing that these sequences did not originate directly from the vaccine. Nevertheless, it is possible that these strains represent drifted viruses that originated from the vaccine strain since numerous vaccine-like isolates were identified after the introduction of Porcilis vaccine in Denmark indicating that the vaccine strain has spread among herds and has become established in Denmark. Since no clinical information was provided it is unclear if these strains are capable of inducing severe clinical disease and it is also unclear if the 74 amino acid deletion can be used as a general marker for Porcilis-like viruses. Interestingly, this region spans the epitope sites ES3 and the putative T-cell epitope and may therefore be prone to changes. This is also supported by the finding of other PRRSV Type 1 viruses harboring deletions in the same region (Fig. 5). One Danish virus harbored a 3 amino acid deletion in ES7 which has not previously been reported. The significance of these epitope sites in nsp2 was confirmed by a study by Chen et al. (2010) showing that deletion of ES3 actually increased the cytolitic activity and showed more vigorous growth kinetics in vitro and produced a higher viral peak load in pigs. The opposite was seen for deletions of ES4 and ES7. The impact of the ES3 deletion in nsp2 of the vaccine strains

should be investigated more thoroughly since it may suggest that the Porcilis PRRS vaccine strain has the ability to replicate more efficiently.

Of the structural proteins, the highest degree of variation was seen in GP3 and GP4. Especially the areas predicted to contain epitopes underwent higher mutation rates and harbored more deletions than those motifs crucial for protein folding and functions, such as cysteine and glycosylation sites. From the deduced amino acid sequences of GP2, GP3, and GP4, 2, 7 and 4 putative N-glycosylation motifs were present in all the Danish sequences, thus only four sites in GP3 and two sites in GP4 were predicted to be N-glycosylated by a neural network.

In conclusion, this study presents for the first time the examination of complete genomes obtained from PRRS Type 1 viruses isolated in a country with very limited import of living animals and boar semen. Only 18 complete genomes (or nearly complete) of PRRSV genotype 1 sequences were as of date available for comparison in public databases, where 10 of the genomes were obtained from viruses isolated in Europe in the years 1991–2007 (Darwich et al., 2011; Van Doorselaere et al., 2011, 2012; Meulenbergh et al., 1993). Thus, the present work adds valuable data on the PRRSV Type 1 viruses circulating in Europe. Characterization of the genetic and antigenic diversity of PRRSV is mandatory for the development and maintenance of diagnostic tools and for development of new vaccine entities.

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